

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

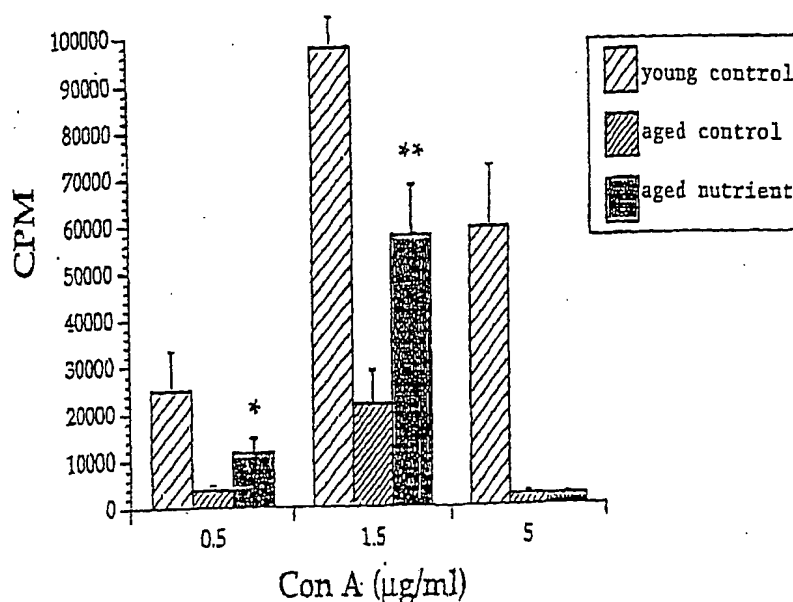


D2

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A23L 1/302, 1/30, 1/305, A61K 31/355, 31/44, 31/20, 45/06		A1	(11) International Publication Number: WO 00/67596
			(43) International Publication Date: 16 November 2000 (16.11.00)
(21) International Application Number: PCT/US00/12300 (22) International Filing Date: 5 May 2000 (05.05.00) (30) Priority Data: 60/133,101 7 May 1999 (07.05.99) US (71) Applicant (for all designated States except US): TRUSTEES OF TUFTS COLLEGE [US/US]; Medford, MA 02155 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): MEYDANI, Simin, Nikbin [US/US]; 650 Centre Street, Newton, MA 02458 (US). MEYDANI, Mohsen [US/US]; 650 Centre Street, Newton, MA 02458 (US). (74) Agents: PIERCE, N., Scott et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).		(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	

(54) Title: IMMUNE STIMULATING DIETARY SUPPLEMENT AND METHODS OF USE THEREOF



(57) Abstract

The immune system of middle aged and elderly individuals is stimulated with a dietary supplement. The dietary supplement includes Vitamin E, Vitamin B6 and conjugated linoleic acid. The dietary supplement can further include glutathione alone or in combination with Vitamin C, folic acid, zinc, selenium, Vitamin D, copper and Vitamin B12. The dietary supplement is administered to middle aged and elderly individuals in a suitable form for consumption by the individual. Suitable forms of consumption can include a snack bar, tablet, capsule, powder, drink, or dairy products.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## IMMUNE STIMULATING DIETARY SUPPLEMENT AND METHODS OF USE THEREOF

### RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial  
5 Number 60/133,101, filed May 7, 1999, the entire teachings of which are  
incorporated herein by reference.

### GOVERNMENT SUPPORT

This invention was made with Government support under 58-1950-9-001  
awarded by the United States Department of Agriculture. The Government has  
10 certain rights in the invention.

### BACKGROUND OF THE INVENTION

Immune responses gradually decline with increasing age. Coincident with a  
decline in immune responses is a concomitant increase in the incidence of tumor  
development, infection and inflammatory diseases in middle aged and elderly  
15 populations of individuals. ("Fundamental Immunology" ed. W.E. Paul, Raven  
Press, NY (1989); Miller, R. A., *Exp. Gerontol.* 29:21-35 (1994). Compromised  
nutritional status can contribute to the impaired immunological state and, hence,  
declining health of aging individuals. Thus, there is a need to develop convenient  
and effective methods that augment the nutritional requirements of middle aged and  
20 elderly individuals, thereby stimulating the immune system to combat disease.

### SUMMARY OF THE INVENTION

The present invention relates to a dietary supplement. It also is directed to a  
method to stimulate the immune system of middle aged and elderly individuals or to  
stimulate proliferation of a lymphocyte by administration of the dietary supplement.

In one embodiment, the dietary supplement comprises Vitamin E, Vitamin B6 and conjugated linoleic acid. In a specific embodiment, the dietary supplement includes Vitamin E in an amount in a range of between about 10 milligrams and about 267 milligrams per milligram of Vitamin B6, and conjugated linoleic acid in an amount in a range of between about 17 milligrams and about 100 milligrams per milligram of Vitamin B6. In another specific embodiment, the dietary supplement further includes glutathione Vitamin C, folic acid, zinc, selenium, Vitamin D, copper, Vitamin B12 and glutathione. Preferably, the dietary supplement includes Vitamin C in an amount in a range of between about 17 milligrams and about 200 milligrams per milligram of Vitamin B6; folic acid in an amount in a range of between about 0.05 milligrams and about 0.2 milligrams per milligram of Vitamin B6; zinc in an amount in a range of between about 1.67 milligrams and about 10 milligrams per milligram of Vitamin B6; selenium in an amount in a range of between about 0.005 milligrams and about 0.02 milligrams per milligram of Vitamin B6; Vitamin D in an amount in a range of between about 0.0008 milligrams and about 0.005 milligrams per milligram of Vitamin B6; copper in an amount in a range of between about 0.00008 milligrams and about 0.0007 milligrams per milligram of Vitamin B6; Vitamin B12 in an amount in a range of between about 0.0002 milligrams and about 0.001 milligrams per milligram of Vitamin B6; and glutathione in an amount in a range of between about 4 milligrams and about 33 milligrams per milligram of Vitamin B6.

In another embodiment, the dietary supplement comprises about 60-800 IU of Vitamin E (such as d- $\alpha$ -tocopherol, dl- $\alpha$ -tocopheryl acetate, dl- $\alpha$ -tocopheryl); about 3-6 mg of Vitamin B6; and about 100-300 mg of conjugated linoleic acid. In another embodiment, the dietary supplement is administered to a middle aged or elderly individuals.

In another embodiment, the dietary supplement further includes about 25-100 mg of glutathione (such as  $\gamma$ -glutamylcysteinylglycine) alone, or in combination with about 100-600 mg of Vitamin C; about 300-600  $\mu$ g of folic acid; about 10-30 mg of zinc; about 30-60  $\mu$ g of selenium; about 5-15  $\mu$ g of Vitamin D; about 0.5-2  $\mu$ g of copper; and about 1-3  $\mu$ g of Vitamin B12.

In yet another embodiment, the invention is a method of stimulating the immune system of an individual comprising formulating the dietary supplement and administering the dietary supplement to the individual.

5 In still another embodiment, the invention is a method of stimulating the proliferation of lymphocytes comprising formulating the dietary supplement and administering the dietary supplement to the lymphocytes. In a preferred embodiment, T-lymphocytes (T-cells) are stimulated to proliferate.

The invention described herein provides an alternative and improved dietary supplement that can conveniently be used to stimulate immune responses in middle  
10 aged and elderly individuals. Thus, administration of the dietary supplement of the present invention can potentially prevent infection-mediated diseases and tumor formation as a result of compromised immune function in individuals, in particular middle aged and elderly individuals.

#### BRIEF DESCRIPTION OF THE FIGURES

15 Figure 1 illustrates the effect of varying dilutions (1/10; 1/5; 1/2.5; 1/1) of the nutrient mixture on Conacavalin A-stimulated splenocyte proliferation in cells isolated from aged C57BL/6 mice.

Figure 2 depicts the body weight (grams) of young mice fed a control diet (young control ---X---), aged mice fed a control diet (aged control ---O---) and aged  
20 mice fed the nutrient mixture (aged treatment ---●---).

Figure 3 illustrates the effect of Conacavalin A on splenocyte proliferation in cells isolated from young and aged mice not fed the nutrient mixture (young control and aged control, respectively) and aged mice fed the nutrient mixture (aged nutrient).  $p < 0.05$  (\*);  $p < 0.01$  (\*\*).

25 Figure 4 illustrates the effect of phytohemagglutinin (PHA) on splenocyte proliferation in cells isolated from young and aged mice not fed the nutrient mixture (young control and aged control, respectively) and aged mice fed the nutrient mixture (aged nutrient).  $p < 0.05$  (\*);  $p < 0.01$  (\*\*).

## DETAILED DESCRIPTION OF THE INVENTION

The features and other details of the invention, either as steps of the invention or as combinations of parts of the invention, will now be more particularly described and pointed out in the claims. It will be understood that the particular  
5   embodiments of the invention are shown by way of illustration and not as limitations of the invention. The principle features of this invention can be employed in various embodiments without departing from the scope of the invention.

The present invention relates to the discovery that a dietary supplement can stimulate the immune system of middle aged and elderly individuals. In particular,  
10   the dietary supplement of the invention stimulates the function of and proliferation of T-lymphocytes (T-cells) of middle aged and elderly individuals. The phrase "dietary supplement" is used interchangeably herein with the phrase "nutrient mixture."

In one embodiment, the dietary supplement comprises Vitamin E, Vitamin  
15   B6 and conjugated linoleic acid. In a specific embodiment, the dietary supplement includes Vitamin E in an amount in a range of between about 10 milligrams and about 267 milligrams per milligram of Vitamin B6, and conjugated linoleic acid in an amount in a range of between about 17 milligrams and about 100 milligrams per milligram of Vitamin B6. In another specific embodiment, the dietary supplement  
20   further includes glutathione Vitamin C, folic acid, zinc, selenium, Vitamin D, copper, Vitamin B12, and glutathione. Preferably, the dietary supplement includes Vitamin C in an amount in a range of between about 17 milligrams and about 200 milligrams per milligram of Vitamin B6; folic acid in an amount in a range of between about 0.05 milligrams and about 0.2 milligrams per milligram of Vitamin  
25   B6; zinc in an amount in a range of between about 1.67 milligrams and about 10 milligrams per milligram of Vitamin B6; selenium in an amount in a range of between about 0.005 milligrams and about 0.02 milligrams per milligram of Vitamin B6; Vitamin D in an amount in a range of between about 0.0008 milligrams and about 0.005 milligrams per milligram of Vitamin B6; copper in an amount in a range  
30   of between about 0.00008 milligrams and about 0.0007 milligrams per milligram of Vitamin B6; Vitamin B12 in an amount in a range of between about 0.0002 milligrams and about 0.001 milligrams per milligram of Vitamin B6; and

glutathione in an amount in a range of between about 4 milligrams and about 33 milligrams per milligram of Vitamin B6.

In another specific embodiment, the dietary supplement comprises about 60-800 IU of Vitamin E; about 3-6 mg of Vitamin B6; and about 100-300 mg of conjugated linoleic acid. In another embodiment, the dietary supplement further includes about 25-100 mg of glutathione alone or in combination with about 100-600 mg of Vitamin C; about 300-600  $\mu$ g of folic acid; about 10-30 mg of zinc; about 30-60  $\mu$ g of selenium; about 5-15  $\mu$ g of Vitamin D; about 0.5-2  $\mu$ g of copper; and about 1-3  $\mu$ g of Vitamin B12.

The dietary supplement is formulated by blending the components (e.g., Vitamin E, Vitamin B6, conjugated linoleic acid, glutathione) to produce a mixture. In a particular embodiment, Vitamin E is d- $\alpha$ -tocopherol, dl- $\alpha$ -tocopheryl acetate or dl- $\alpha$ -tocopheryl and glutathione is  $\gamma$ -glutamylcysteinylglycine. Methods to mix the components of the dietary supplement can include, for example, the addition of components of relatively lower concentration or quantity (e.g., selenium, copper) to components of relatively higher concentration or quantity (e.g., Vitamin C, conjugated linoleic acid, glutathione). The dietary supplement can be stored at ambient temperature (e.g., 18-25°C) or at cooler temperatures, for example, in a refrigerator (e.g., 4°C).

In one embodiment, the dietary supplement is formulated by blending Vitamin C, Vitamin B6, zinc, folic acid, selenium, copper, Vitamin D, Vitamin B12 and glutathione to prepare a stock mixture of the dietary supplement. The fat soluble components, Vitamin E and conjugated linoleic acid, are then added to the stock mixture prior to use (e.g., *in vitro* addition to lymphocytes). In another embodiment the dietary supplement is formulated by simultaneously blending all of the components in any order.

As defined herein, a "middle aged" individual refers to an individual who is at an age between about 40% to about 60% of the expected lifespan of the individual. For example, a middle-aged human individual is a human between the age of about 45 to about 65 years old. Likewise, a middle-aged C57BL/6NCrIBr mouse is a mouse about 14 to about 16 months of age. An "elderly" individual refers to an individual who is an age in a range of between about 65% to about 100%

of the expected lifespan of the individual. For example, an elderly human individual is a human greater than about 65 years of age. Similarly, an elderly C57BL/6NCrlBr mouse is a mouse greater than about 16 months of age, such as a mouse of about 24 months of age.

5           The term "individual" is used to refer to any mammal. For example, the mammal can be a primate mammal (e.g., human, monkey, baboon) domesticated mammal (e.g., dog, cat), ruminant mammal (e.g., cow, sheep, horse) or rodent mammal (e.g., mouse, rat, guinea pig). In particular, the dietary supplement is used to stimulate the immune system of human mammals. It is also envisioned that the  
10 dietary supplement can be used in veterinary practice to stimulate the immune system of other mammals such as dogs and cats.

In a method of the invention, the dietary supplements are administered to middle aged and elderly individuals to stimulate the immune system. The supplement is administered to the individuals by a means suitable for consumption,  
15 such as a tablet or capsule which can be easily swallowed, chewed, or dissolved. Additionally, or alternatively, the dietary supplement can be formulated into a powder or liquid for convenient addition to drinks, baked goods, dairy products or other food stuffs. The dietary supplement can also be consumed in the form of a snack bar, drink or lozenge. The supplement preferably is consumed on a daily  
20 basis, e.g., once a day. The dietary supplement can also be consumed multiple (greater than one) times a day.

The term "stimulate", as used herein, refers to any augmentation, increase, or enhancement of an immunological indice in the individual following consumption of the dietary supplement. In a preferred embodiment, the dietary supplement  
25 stimulates T-lymphocytes (also referred to herein as T-cells) function. In a particularly preferred embodiment, T-lymphocyte proliferation is the stimulated immune response.

Optionally, stimulation of the immune system of the individual being administered the dietary supplement can be monitored. Methods to assess immune  
30 system stimulation are routine and well known to those skilled in the art. For example, indices of immune stimulation can be assessed in peripheral blood samples obtained from individuals consuming the dietary supplement by monitoring the



proliferation of phytohemagglutinin-(PHA) or concanavalin A(ConA)-stimulated lymphocytes. Additionally, or alternatively, the levels of immunoglobulins (e.g., IgA, IgG and IgM), antibody titers against known vaccines or antigens, concentrations and synthesis of cytokines (e.g., IL-2) and the number and type of leucocytes (e.g., polymorphonuclear or mononuclear) can be determined in peripheral blood samples. Methods to assay immune stimulation can also include evaluations of delayed-type hyper-sensitivity skin responses, resistance to infectious diseases, natural killer cell activity, or any combination thereof. Exemplary techniques to assess immune system stimulation can be found, for example, in

10 Kennes, B., *et al.*, *Gerontology* 29:305-310 (1983); Meydani, S. N., *et al.*, *Mech. Aging and Develop.* 34:191-201, (1986); Meydani, S. N., *et al.*, *Ann. N.Y. Acad. Sci.* 570:283-290, (1989); Meydani, S. N., *et al.*, *Am. J. Clin. Nutr.* 52:557-563, (1990); Meydani, S. N., *et al.*, *Am. J. Clin. Nutr.* 53:1275-1280 (1991); Wu, D., *et al.*, *J. Nutr.* 124:655-663, (1994); Meydani, S. N., *et al.*, *JAMA* 277:1380-1386 (1997);

15 Hayek, M. G., *et al.*, *J. Infect. Dis.* 17:273-276 (1997), the teachings of all of which are hereby incorporated by reference in their entirety).

Immune responsive indices following treatment with the dietary supplement of the invention can be compared with indices prior to treatment. If necessary, the dose and frequency of administration of the dietary supplement can be increased to further stimulate the immune system. Similarly, the dose and frequency of administration of the dietary supplement can be decreased following stimulation of the immune system (e.g., T-lymphocyte proliferation).

20

In another embodiment, the invention is a method of stimulating the proliferation of lymphocytes. The dietary supplement is formulated and administered to the lymphocytes (e.g., T-lymphocytes, B-lymphocytes). In a preferred embodiment, T lymphocytes are stimulated to proliferate. Stimulation of T-lymphocytes is a well recognized indice of T-lymphocyte function, Beharka, A., *et al.*, *Methods in Enzymology* 282:247-263 (1997); "Fundamental Immunology", ed. W. E. Paul, Raven Press, NY: (1989); "Methods in Cellular Immunology", ed.

25

30 Fernandez, R., *et al.*, CRC Press, Boca Raton, FL (1995), the teachings of all of which are hereby incorporated by reference in their entirety. T-lymphocytes can be stimulated to proliferate by the methods of the invention, for example, by

stimulating proliferation in a mammal *in vivo* following consumption of the dietary supplement. Similarly, T-lymphocytes can be obtained from the mammal (e.g., from the spleen or peripheral blood) and exposed to the dietary supplement *in vitro* to stimulate proliferation; this stimulation can be an assessment of the effect of the dietary supplement on T-lymphocyte function *in vivo*. T-lymphocytes obtained from the mammal can be a population or collection of cells which consist essentially of T-lymphocytes, or a population of cells that consist of T-lymphocytes as well as other cell types (e.g., B-lymphocytes, fibroblast, red blood cells). Suitable methods to assess T-cell proliferation are well known to one of skill in the art (See, for example, Wu, *et al.*, *J. Nutr.* 128: 193-197 (1998)), the teachings of which are hereby incorporated by reference in its entirety).

The present invention is further illustrated by the following examples, which are not intended to be limiting in anyway.

#### EXAMPLE 1: *IN VITRO* EFFECTS OF THE NUTRIENT MIXTURE ON CELLS ISOLATED FROM AGED ANIMALS

##### Materials and Methods:

A stock mix for use in *in vitro* experiments was formulated by blending the components in the units and order as listed in Table 1. RPMI 1640 media (BioWhitaker, Walkerville, M.D.) was used to prepare the stock mixture and for dilution of components added to the stock mixture (vitamin E and conjugated linoleic acid). The stock mixture was stored at ambient temperature (e.g., 18-25°C). For *in vitro* experiments, the stock mixture was prepared fresh prior to dilution in RPMI 1640 media.

TABLE 1: Composition of the Stock Mixture for *In Vitro* Experiments

	<u>Component</u>	<u>Units</u>
	Vitamin C	500 mg
	Vitamin B6	5 mg
5	Zinc	20 mg
	Folic Acid	500 µg
	Selenium	50 µg
	Copper	1.4 µg
	Vitamin D	10 µg
10	Vitamin B12	1 µg
	Glutathione	25 mg

The stock mixture was dissolved in RPMI 1640 media to prepare a solution containing 12.5 milligrams of the stock mixture per milliliter of RPMI 1640 media. For example, 62.5 mg of the stock mixture was dissolved in 5 ml of RPMI 1640 media which was then serially diluted to prepare a 12.5 microgram of the stock solution per milliliter of RPMI 1640. The concentrations of each component in a 12.5 µg/ml of the stock mixture in RPMI 1640 media are listed in Table 2.

A stock solution of conjugated linoleic acid (1mg/ml) was prepared by dissolving 25 µl of a 20 mg conjugated linoleic acid per ml stock solution in 475 µl of RPMI 1640 media. A 2.5 µg/ml solution was then prepared by adding 50 µl of the 1 mg/ml solution to 19.95 ml of RPMI 1640 media.

Vitamin E (d-α-tocopherol) was prepared as a 0.5 µg/ml solution. Initially a 2 mg/ml stock solution was prepared by dissolving 29.4 µl of a d-α-tocopherol stock (34 mg/ml) into 471.6 µl of fetal bovine serum (FBS). Fetal bovine serum was used in these experiments since *in vivo* vitamin E is incorporated into serum lipoproteins before it can be delivered to cells. Fetal bovine serum contains lipoproteins, therefore it is used as a vehicle to deliver vitamin E to cells in *in vitro* splenocyte cultures. The vitamin E dissolved in fetal bovine serum was incubated at 37°C for fifteen minutes to allow incorporation of vitamin E into the fetal bovine serum lipoproteins. Following the incubation, a 5 µg/ml solution of vitamin E was prepared by dissolving 50 µl of a 2 mg/ml solution into 19.95 ml of RPMI 1640.

TABLE 2: Units of Each Component in a 12.5  $\mu\text{g/ml}$  of the Stock Mixture for *In Vitro* Experiments

	<u>Component</u>	<u>Units <math>\mu\text{g/ml}</math></u>
5	Vitamin C	11.89
	Vitamin B6	0.1189
	Zinc	0.4756
	Folic Acid	0.0119
	Selenium	0.00119
10	Copper	0.000034
	Vitamin D	0.00024
	Vitamin B12	0.000024
	Glutathione	0.59460

The mixture listed in Table 2 was supplemented with conjugated linoleic acid and vitamin E so that the concentrations of each of the components in the nutrient mixture added to cells *in vitro* were as listed in Table 3. For example, 5 ml of the mixture in Table 2 (12.5  $\mu\text{g/ml}$ ) was admixed with 2.5 ml of vitamin E (5  $\mu\text{g/ml}$  stock solution) and 2.5 ml of conjugated linoleic acid (2.5  $\mu\text{g/ml}$  stock solution).

TABLE 3: Concentration of Nutrients Added to Cells in *In Vitro* Cultures

	<u>Component</u>	<u>Units <math>\mu\text{g/ml}</math></u>	<u>Units (ng/ml) Added to Cell Cultures (<math>1 \times 10^6</math> cells/ml)</u>
20	Vitamin C	5.945	2378
	Vitamin B6	0.0594	23.76
	Zinc	0.2378	95.12
	Folic Acid	0.00595	2.38
	Selenium	0.000595	0.238
25	Copper	0.000017	0.0068
	Vitamin D	0.00012	0.048
	Vitamin B12	0.000012	0.0048
	Glutathione	0.297301	118.92
30	Vitamin E	0.625	250
	Conjugated linoleic acid	0.312	124.8

Aged (24 months of age) C57BL/6NCrIBR mice ( $n=6$ ) were used in these experiments. The mean life span of a C57BL6 mouse is about 28 months. Aged mice were fed a commercial chow diet of Commercial Rodent Diet, Teklad 7012 (Teklad, Madison, WI).

Splenocytes were obtained from aged mice, cultured in the presence or absence of the nutrient mixture and their ability to proliferate in response to the T cell mitogen, ConA was determined as previously described (See, for example, Wu, *et al.*, *J. Nutr.* 128: 193-197 (1998), the teachings of which are hereby incorporated

by reference in their entirety). Briefly, mice were euthanized with CO<sub>2</sub> asphyxiation, and their spleens were removed aseptically and placed in sterile RPMI 1640 media (BioWhitaker, Walkerville, MD) supplemented with 25 mM HEPES/L, 2mM glutamine/L, 1X10<sup>5</sup> units penicillin/L and 100 mg streptomycin/L.

5           A 100 µl aliquot of the mixture in the concentrations listed in Table 3 was added to 100 µl aliquot of splenocyte cell cultures (2.5 x 10<sup>6</sup> ml cells) and incubated for four hours. The nutrient mixture was diluted 1/1; 1/2.5; 1/5 and 1/10 (volume:volume) with RPMI 1640 culture medium.

          After the four hour incubation (37°C, 5% CO<sub>2</sub> and 95% humidity), Con A  
10   (1.5 mg/L of RPMI 1640 medium) was added to splenocyte cultures. Con A and PHA only stimulate T cells to proliferate ("Fundamental Immunology", ed. W.E. Paul, Raven Press, NY (1989); "Methods in Cellular Immunology", eds. Fernandez, R., *et al.*, CRC Press, Boca Raton, FL (1995), the teachings of both of which are hereby incorporated by reference in their entirety). Therefore, the proliferative  
15   response to Con A and PHA is referred to as a T-cell mediated response. The cells were cultured for an additional 72 hours (37°C). Con A and PHA were used in these experiments because lymphocytes from aged mice and humans have been shown to have a significantly lower proliferative response to the T cell mitogens ConA and PHA (Miller, R.A., *Exp. Gerontol.* 29:21-35 (1994), the teachings of which are  
20   hereby incorporated by reference in its entirety). In addition, a mitogenic response induced by ConA is used as a measure of T cell function (Beharka, A., *et al.*, *Methods in Enzymology* 282:247-263 (1997), the teachings of which are hereby incorporated by reference in their entirety).

          After 68 hours of the 72 hour culture period, [<sup>3</sup>H]-thymidine (18.5 micro Bq)  
25   was added to culture media. After 4 hours of incubation with [<sup>3</sup>H]-thymidine, cells were harvested onto glass fiber filter mats by a TOMTEC harvester. Cell proliferation (also referred to as a proliferative response) was defined as the amount of [<sup>3</sup>H] thymidine incorporation into DNA over the 4 hour period as measured by liquid scintillation counting in a 1205 BETAPLATE counter. The BETAPLATE  
30   counter had an efficiency of more than 50% for <sup>3</sup>H. Data were expressed as counts per minute (cpm). Counts per minute was calculated as the cpm of mitogen-

-12-

stimulated cultures minus the cpm of cultures without mitogen. The counts per minute were expressed as the mean  $\pm$  SEM.

### Results:

As shown in Figure 1, the *in vitro* addition of nutrient mixture to *in vitro* cultured splenocytes obtained from aged mice significantly increased the proliferative response of T cells to the mitogen ConA. Splenocytes obtained from aged mice and incubated in the nutrient mixture proliferated 5 fold more in response to ConA stimulation (at 1/1 dilution) than splenocytes cultured in the absence of the nutrient mixture (control). An increased proliferation of T-lymphocytes in response to Con A indicated a measure of improvement in the immune response.

The effect of the nutrient mixture on splenocyte proliferation and, thus, the immune response of cells was dose dependent. As the nutrient mixture was diluted, the proliferative response of splenocytes decreased (Figure 1). Since proliferation of T cells in response to ConA has been shown to be significantly reduced in aged mice and humans (Miller, R.A., *Exp. Gerontol* 29:21-35 (1994), the teachings of which are incorporated herein in their entirety), these results show that the nutrient mixture can significantly improve the immune response in the aged mammal.

## EXAMPLE 2: FEEDING THE NUTRIENT MIXTURE TO AGED MICE SIGNIFICANTLY IMPROVED T CELL MEDIATED FUNCTION

### 20 Materials and Methods

Specific Pathogen-Free young male (5 months old) and aged male (24 months old) C57BL/6NCrIBR mice were randomly assigned to a diet containing the National Research Council (NRC)-recommended level of all nutrients (control diet) or the control diet supplemented with the nutrient mixture. The composition of the control diet was based on AIN 93 (Reeves, P.G., *et al.*, *J. Nutri* 123: 1939-1951 (1993), the teachings of which are hereby incorporated by reference in its entirety). The components and the concentration of components in the control diet and diet supplemented with the nutrient mixture are shown in Table 4. The concentration of the components in the nutrient mixture fed to mice are listed in Table 4.

The average caloric intake of a human is about 2100 Kcal per day (Recommended Daily Allowance, RDA). The average caloric intake of a mouse is about 13.84 Kcal per day. The 13.84 Kcal per day caloric intake is equivalent to about 3.8 g of food in a mouse diet per day. (Meydani, S. N., *et al.*, *J. Nutr.* 121:547-555 (1991), the teachings of which are incorporated herein in their entirety). For example, in a human the amount of Vitamin E in a formula is 200 International Units (IU) (See Table 4) for a 2100 Kcal diet. To calculate the "mouse equivalent" dose or the amount to be added to a mouse diet to give a comparable amount of Vitamin E based on the 13.84 Kcal per daily caloric intake of a mouse the following calculations were employed:

$$\frac{200 \text{ IU Vitamin E} \times 13.84 \text{ Kcal}}{2100 \text{ Kcal}} = 1.318 \text{ IU Vitamin E for a 13.84 Kcal diet of a mouse}$$

Since 13.84 Kcal of an average caloric intake in the mouse is equivalent to about 3.8 grams of a mouse diet (also referred to as food), the amount of Vitamin E added per kg (1000g) of mouse food was calculated as follows:

$$\frac{1.318 \text{ IU Vitamin E} \times 1000 \text{ g}}{3.8 \text{ g}} = 347 \text{ IU Vitamin E per kg of food}$$

Employing the above strategy, the nutrient mixture was prepared containing the concentrations of each component as listed in Table 4. An amount of the mixture was admixed with and stirred into the control diet to achieve the concentrations listed in Table 4. Food and water were provided *ad libitum* to the mice.

TABLE 4: Components in a Human Diet, Mouse Diet and Diet Fed to Mice in *In vivo* Experiments the Nutrient Mixture.

		Human	Mouse	Control Diet	Control Diet Supplemented With the Nutrient Mixture
5	<u>Component</u>	<u>Units/day</u>	<u>Units /kg diet</u>	<u>Units/kg diet</u>	<u>Units: IU/kg food or mg/kg food</u>
	Vitamin E	200 IU	347 IU*	30 IU	377 IU
	Vitamin B6	5.0 mg	8.6 mg	7.0 mg	15.67 mg
	Conjugated linoleic acid	100 mg	174 mg	0	174.00 mg
10	Glutathione	25 mg	44 mg	0	44.0 mg
	Vitamin C	500 mg	868 µg	0	868.00 mg
	Folic Acid	500 µg	87 µg	2.0 mg	2.87 mg
	Zinc	20 mg	34.8 mg	57.8 mg	92.60 mg
	Selenium	50 µg	87 µg	358.7 mg	358.787 mg
15	Vitamin D	10 µg	17.4 µg	0.0173 mg	0.0347 mg
	Copper	1.4 µg	2.4 µg	10.5 mg	10.50243 mg
	Vitamin B12	1 µg	1.74 µg	0.025 mg	0.02674 mg

\*dl - α - tocopheryl acetate

20 Mice were randomly divided into three treatment groups. Group I mice (n=12) were young mice (5 months of age) fed a control diet. Group II mice (n=12) were aged mice (24 months old) who were fed a control diet for 4.5 weeks. Group II mice are also referred to as aged control mice. Group III mice (n=12) were aged mice (24 months) who were fed a control diet supplemented with the nutrient  
25 mixture. Group III mice were also referred to as aged nutrient mice. Group II and III aged mice were fed either the control or the diet supplemented with the nutrient mixture, respectively, for 4 ½ weeks.

Mice were individually housed in filtered cages in an environmentally controlled atmosphere (temperature 23°C, 45% relative humidity) with fifteen air  
30 changes of 100% fresh-filtered air per hour and a 12/12-hour light-dark cycle. Mice were weighed weekly.

Food intake was recorded daily. Food intake was calculated by subtracting the weight of left over food in each cage (one mouse per cage) from the food provided. Specialized food containers, which restrict food spillage, were used to  
35 reduce the loss of food due to digging.

To harvest splenocytes mice were euthanized by CO<sub>2</sub> asphyxiation and spleens excised aseptically. Single splenocyte cell suspensions were prepared and cultured for lymphocyte proliferation as described in Example 1 using established procedures (See, for example, Wu, *et al.*, *J. Nutr.* 128: 193-197 (1998), the teachings



of which are hereby incorporated by reference in their entirety). Details of culture conditions were similar to those described in Example 1, except the nutrient mixture was not added to *in vitro* cultures.

#### Results:

5 As shown in Figure 2, there was no difference in weight gain between the treatment groups.

Mice in all the treatment groups consumed about 3.8 g of food per day. There was no difference in food intake between aged mice fed a control diet or a supplemented diet or young mice fed a control diet.

10 Based on the daily food intake of about 3.8 g per day, Table 5 depicts about the concentration of each nutrient consumed by mice in each of the treatment groups.

TABLE 5. Dietary Intake of Nutrients.

15	<u>Component</u>	Units (mg)	Units (mg)
		<u>Group III</u>	<u>Groups I and Group II</u>
	Vitamin E*	1.4326*	0.114*
	Vitamin B6	0.059	0.0266
	Conjugated linoleic acid	0.661	0
	Glutathione	0.167	0
20	Vitamin C	3.298	0
	Folic Acid	0.011	0.0076
	Zinc	0.352	0.21964
	Selenium	1.3634	1.3631
	Vitamin D	0.000132	0.00007
25	Copper	0.03991	0.03990
	Vitamin B12	0.0001016	0.000095

\*dl -  $\alpha$  - tocopheryl acetate

Figures 3 and 4 show the proliferative response of splenocytes to varying concentrations of the T cell mitogens ConA and PHA, respectively. Values are expressed as the mean  $\pm$  SEM for twelve individual determinations. A proliferative response was depicted as the counts per minute (CPM) in *in vitro* assays as described in Example 1.

Aged mice fed the control diet had a significantly lower proliferative response to the T cell mitogens ConA (Figure 3) and PHA (Figure 4) compared to young mice fed the control diet. Whereas the proliferative response in splenocytes

isolated from aged mice fed a diet supplemented with the nutrient mixture was significantly greater than the proliferative response of splenocytes isolated from aged mice fed a control diet (Figures 3 and 4). An improvement in *in vitro* proliferation response of splenocytes to T-cell mitogens indicates an improvement (Beharka, A., *et al.*, *Methods in Enzymology* 282:247-263 (1997), the teachings of which are hereby incorporated by reference in their entirety).

These *in vitro* and *in vivo* data show that the nutrient mixture can significantly improve T cell-mediated function in the aged mice. T cell-mediated functions can be functions that are dependent on physiological properties of T-cells, for example, the ability of T-cells to proliferate in response to T-cell mitogens, to produce cytokines, the ability to fight viral infections, the ability to fight bacterial infections, or the ability to attack or fight tumors. ("Fundamental Immunology", ed. W.E. Paul, Raven Press, NY (1989), the teachings of which are hereby incorporated by reference in their entirety). The mouse is an excellent model system for evaluating the effects of nutrients on immune responses in mammals, including humans. For example, vitamin E supplementation to old C576/6NCrlBr mice increased T cell function, vitamin E supplementation to the elderly human improved similar immunological responses (e.g. T-cell mediated function) (Meydani, *et al.*, *Mech. Aging and Develop.* 34:191-201 (1986); Meydani, S. N., *et al.*, *Am. J. Clin. Nutri.* 53:1275-1280 (1991), the teachings of both of which are hereby incorporated by reference in their entirety). Thus, these data strongly suggest that supplementing humans with the nutrient mixture of the invention can significantly improve immune responses in humans.

T cell-mediated function decreases with aging and contributes to the increased incidence of infectious and neoplastic diseases in the aged mammal. Thus, aged mammals can benefit from consumption of this nutrient mixture to stimulate T-cell function thereby decreasing the incidence and severity of infectious and neoplastic diseases in the aged mammal.

When a foreign invader, such as bacteria or a virus enters the body of a mammal (or human) the number of T-cells in the body proliferate to combat the foreign invader. When lymphocytes encounter the foreign invader, they proliferate, a phenomenon referred to as clonal expansion. Clonal expansion enables

lymphocytes to duplicate thereby producing many similar lymphocytes in a short period of time to overcome the foreign invader and rid the foreign invader from the body. Thus, the ability of lymphocytes (e.g., T-lymphocytes) to proliferate in response to an antigen or a mitogen (e.g. ConA, PHA) *in vitro* was used to assess the

5 ability of lymphocytes to proliferate. Lymphocytes obtained from an aging mammal, in particular T-lymphocytes, lose their ability to proliferate and, thus, the ability to combat infection in the aged mammal (e.g. rodents, primates, dogs, humans) is decreased. This decreased ability to combat infection is a characteristic of lymphocytes in aged mammals. Thus, the ability of the nutrient mixture of the

10 invention to improve the ability of T-lymphocytes to proliferate is a significant advantage for the elderly population to combat disease including, for example, infections and immune compromised states such as neoplastic disease.

#### EQUIVALENTS

While this invention has been particularly shown and described with

15 references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

## CLAIMS

What is claimed is:

1. A dietary supplement, comprising:
  - a) Vitamin E;
  - 5 b) Vitamin B6; and
  - c) conjugated linoleic acid.
2. The dietary supplement of Claim 1, wherein the amount of Vitamin E present is in a range of between about 10 and about 267 milligrams per milligram of Vitamin B6.
- 10 3. The dietary supplement of Claim 2, wherein the amount of conjugated linoleic acid present is in a range of between about 17 milligrams of about 100 milligrams per milligram of Vitamin B6.
4. The dietary supplement of Claim 3, further including:
  - a) Vitamin C;
  - 15 b) folic acid;
  - c) zinc;
  - d) selenium;
  - e) Vitamin D;
  - f) copper;
  - 20 g) Vitamin B12; and
  - h) glutathione.
5. The dietary supplement of Claim 4, wherein:
  - a) the amount of Vitamin C present is in a range of between about 17 milligrams and about 200 milligrams per milligram of Vitamin B6;
  - 25 b) the amount of folic acid present is in a range of between about 0.05 milligrams and about 0.2 milligrams per milligram of Vitamin B6;

-19-

- 5 c) the amount of zinc present is in a range of between about 1.67 milligrams and about 10 milligrams per milligram of Vitamin B6;
- d) the amount of selenium present is in a range of between about 0.005 milligrams and about 0.02 milligrams per milligram of Vitamin B6;
- 10 e) the amount of Vitamin D present is in a range of between about 0.0008 milligrams and about 0.005 milligrams per milligram of Vitamin B6;
- f) the amount of copper present is in a range of between about 0.00008 milligrams and about 0.0007 milligrams per milligram of Vitamin B6;
- 15 g) the amount of Vitamin B12 present is in a range of between about 0.0002 milligrams and about 0.001 milligrams per milligram of Vitamin B6; and
- h) the amount of glutathione is in a range of between about 4 milligrams and about 33 milligrams per milligram of Vitamin B6.
6. The dietary supplement of Claim 3, wherein the amount of Vitamin E present is in a range of between about 60 and about 800 International Units.
7. The dietary supplement of Claim 6, wherein the amount of Vitamin B6 present is in a range of between about 3 milligrams and about 6 milligrams.
- 20 8. The dietary supplement of Claim 7, wherein the amount of conjugated linoleic acid present is in a range of between about 100 milligrams and about 300 milligrams.
9. The dietary supplement of Claim 8, wherein said dietary supplement further includes glutathione in an amount in a range of between about 25 milligrams and about 100 milligrams.
- 25

-20-

10. The dietary supplement of Claim 9, further including:
  - a) about 100-600 mg of Vitamin C;
  - b) about 300-600 µg of folic acid;
  - c) about 10-30 mg of zinc;
  - 5 d) about 30-60 µg of selenium;
  - e) about 5-15 µg of Vitamin D;
  - f) about 0.5-2 µg of copper; and
  - g) about 1-3 µg of Vitamin B12.
11. A dietary supplement, comprising:
  - 10 a) about 60-800 IU of Vitamin E;
  - b) about 3-6 mg of Vitamin B6;
  - c) about 100-300 mg of conjugated linoleic acid; and
  - d) about 25-100 mg of glutathione.
12. The method of stimulating the immune system of an individual, comprising  
15 the steps of:
  - a) formulating the dietary supplement that includes Vitamin E, Vitamin B6 and conjugated linoleic acid; and
  - b) administering said dietary supplement to said individual.
13. The method of Claim 12, wherein the dietary supplement is administered to  
20 an individual that is in a range of between about 45 and about 65 years of age.
14. A method of Claim 12, wherein the dietary supplement is administered to an individual that is greater than about 65 years of age.
15. The method of Claim 12, wherein the dietary supplement further includes  
25 glutathione.

16. A method of stimulating proliferation of a lymphocyte, comprising the steps of:
- a) formulating a dietary supplement that includes Vitamin E, Vitamin B6, and conjugated linoleic acid; and
  - 5 b) administering said dietary supplement to said lymphocyte.
17. The method of Claim 15, wherein the dietary supplement is a T-cell lymphocyte.
18. The method of Claim 15, wherein the dietary supplement further includes glutathione.
- 10 19. A method of stimulating the immune system of an individual, comprising the steps of:
- a) formulating a dietary supplement that includes about 60-800 IU of Vitamin E; about 3-6 mg of Vitamin B6; and about 100-300 mg of conjugated linoleic acid; and
  - 15 b) administering said dietary supplement to said individual.
20. The method of Claim 19, wherein the dietary supplement is administered to an individual that is middle-aged.
21. The method of Claim 19, wherein the dietary supplement is administered to an individual that is elderly.
- 20 22. A method of stimulating the immune system of an individual, comprising the steps of:
- a) formulating a dietary supplement that includes about 60-800 IU of Vitamin E; about 3-6 mg of Vitamin B6; about 100-300 mg of conjugated linoleic acid; and about 25-100 mg of glutathione; and
  - 25 b) administering said dietary supplement to said individual.

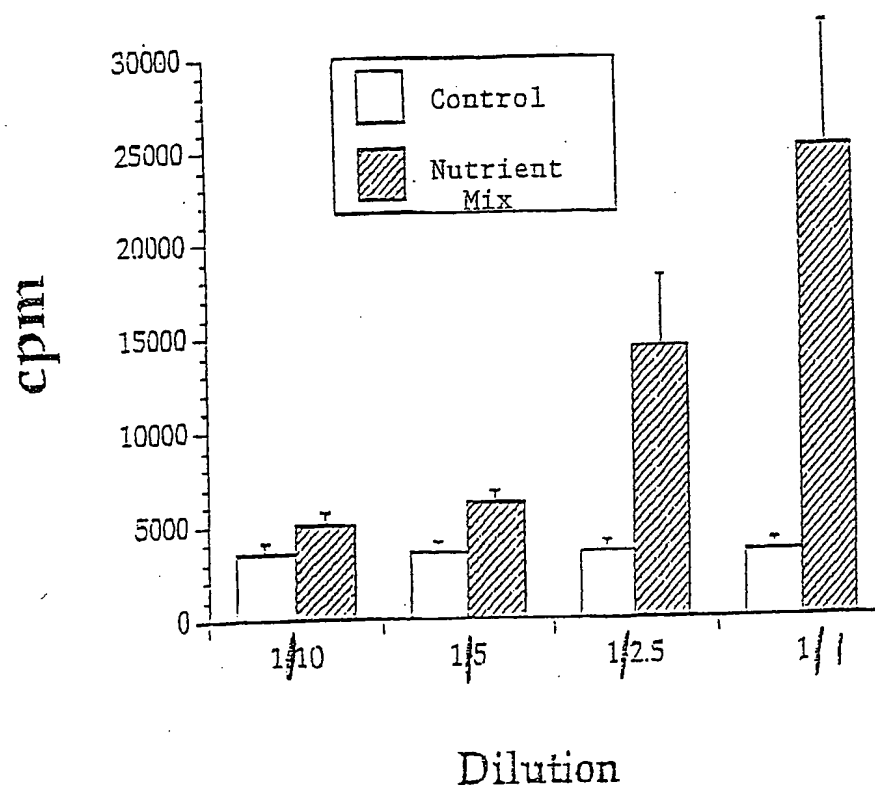
23. The method of Claim 22, wherein the dietary supplement is administered to an individual that is middle-aged.
24. The method of Claim 22, wherein the dietary supplement is administered to an individual that is elderly.
- 5 25. A method of stimulating the immune system of an individual, comprising the steps of:
- 10 a) formulating a dietary supplement comprising about 60-800 IU of Vitamin E; about 3-6 mg of Vitamin B6; about 100-300 mg of conjugated linoleic acid; about 25-100 mg of glutathione; about 100-600 mg of Vitamin C; about 300-600 µg of folic acid; about 10-30 mg of zinc; about 30-60 µg of selenium; about 5-15 µg of Vitamin D; about 0.5-2 µg of copper; and about 1-3 µg of Vitamin B12; and
- b) administering said dietary supplement to said individual.
- 15 26. The method of Claim 25, wherein the dietary supplement is administered to an individual that is middle-aged.
27. The method of Claim 25, wherein the dietary supplement is administered to an individual that is elderly.
28. A method of stimulating proliferation of a lymphocyte, comprising the steps of:
- 20 a) formulating a dietary supplement that includes about 60-800 IU of Vitamin E; about 3-6 mg of Vitamin B6; and about 100-300 mg of conjugated linoleic acid; and
- b) administering said dietary supplement to said lymphocyte.
29. The method of Claim 28, wherein the dietary supplement is administered to a T-cell lymphocyte.
- 25



30. A method of stimulating proliferation of a lymphocyte, comprising the steps of:
- 5 a) formulating a dietary supplement that includes about 60-800 IU of Vitamin E; about 3-6 mg of Vitamin B6; about 100-300 mg of conjugated linoleic acid; and about 25-100 mg of glutathione; and
- b) administering said dietary supplement to said lymphocyte.
31. The method of Claim 30, wherein the dietary supplement is administered to a T-cell lymphocyte.
32. A method of stimulating proliferation of a lymphocyte, comprising the steps of:
- 10 a) formulating a dietary supplement comprising about 60-800 IU of Vitamin E; about 3-6 mg of Vitamin B6; about 100-300 mg of conjugated linoleic acid; about 25-100 mg of glutathione; about 100-600 mg of Vitamin C; about 300-600  $\mu$ g of folic acid; about 10-30
- 15 mg of zinc; about 30-60  $\mu$ g of selenium; about 5-15  $\mu$ g of Vitamin D; about 0.5-2  $\mu$ g of copper; and about 1-3  $\mu$ g of Vitamin B12; and
- b) administering said dietary supplement to said lymphocyte.
33. The method of Claim 32, wherein the dietary supplement is administered to a T-cell lymphocyte.

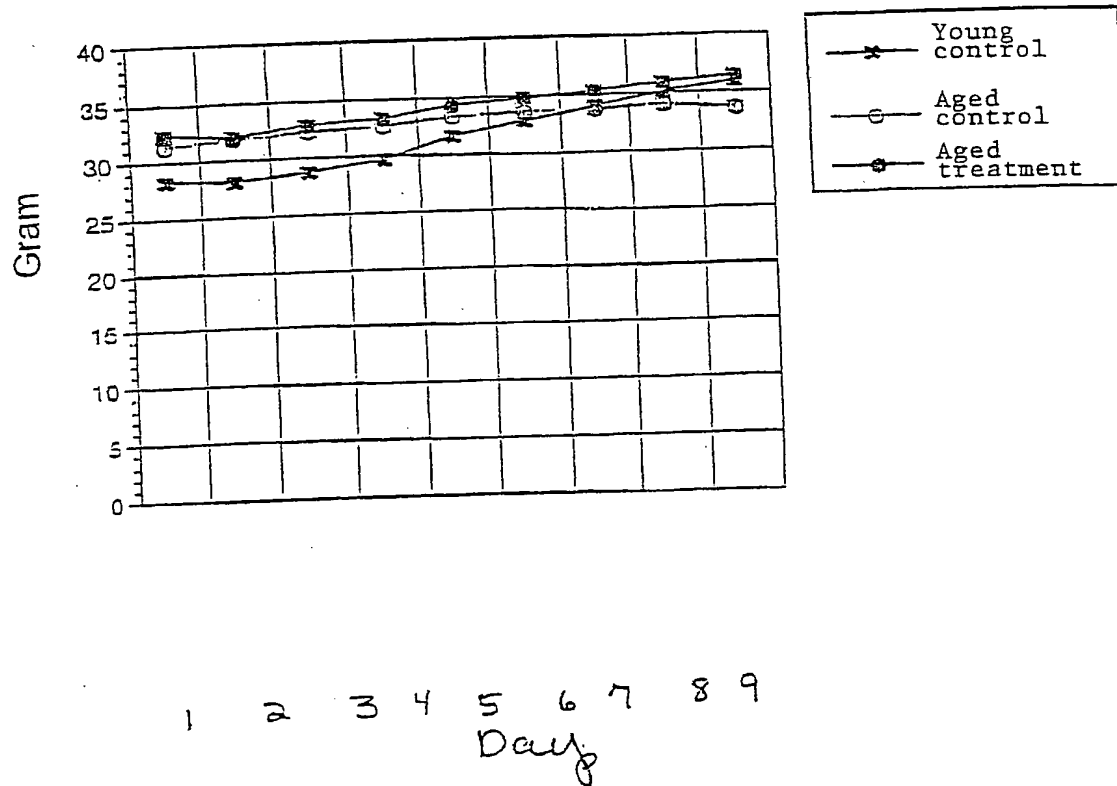
1/4

Figure 1



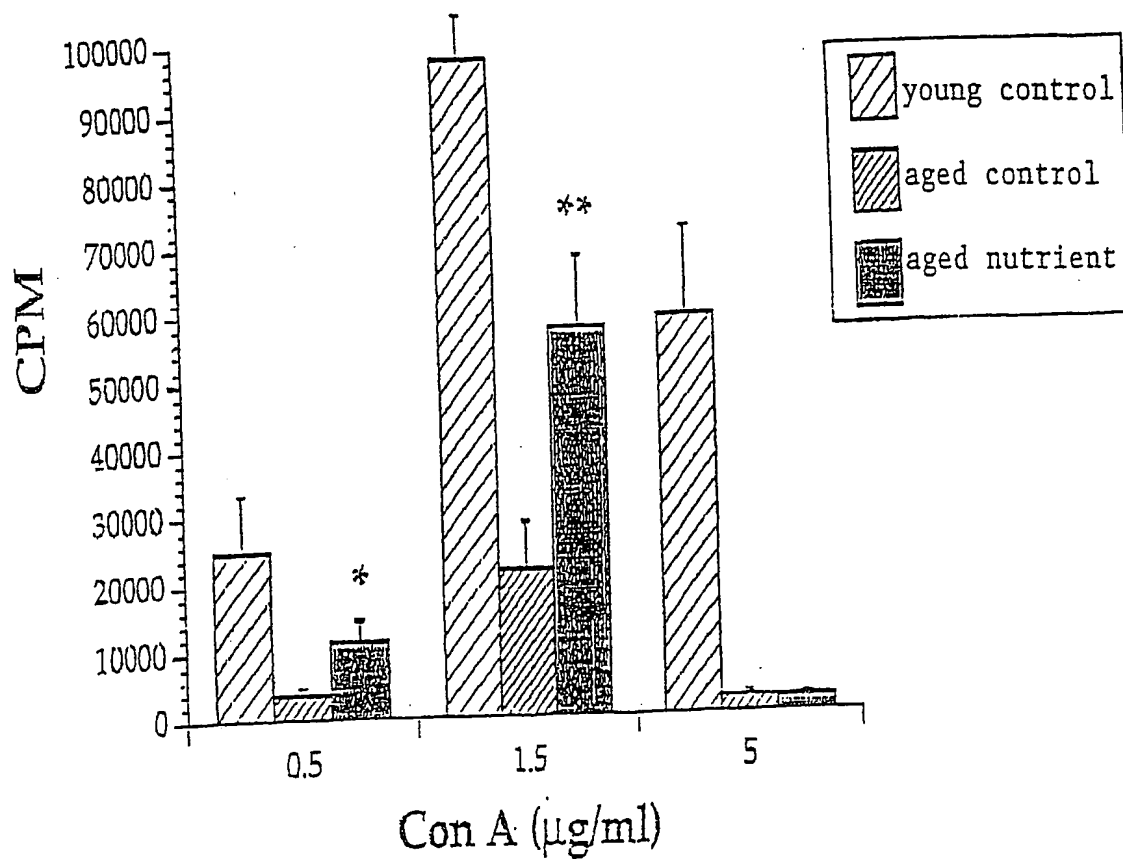
2/4

Figure 2



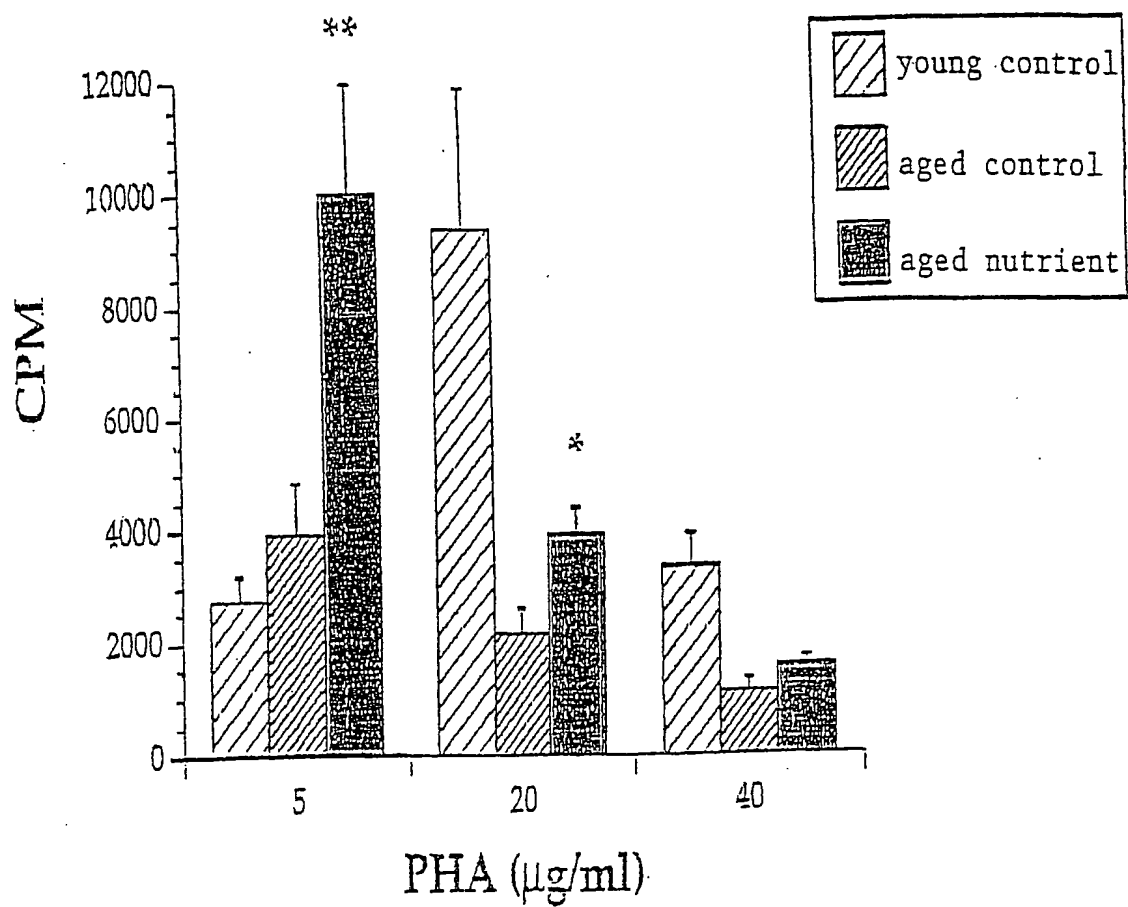
3/4

Figure 3



4/4

Figure 4



# INTERNATIONAL SEARCH REPORT

national Application No

PCT/US 00/12300

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A23L1/302 A23L1/30 A23L1/305 A61K31/355 A61K31/44  
A61K31/20 A61K45/06

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A23L A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 814 663 A (COOK ET AL.) 29 September 1998 (1998-09-29)  example 4 claims	1-3, 12-14, 16,17
1	<p>-----</p> <p>"1989 Recommended Dietary Intakes" INTERNET. 'Online! 2000. XP002148324 Retrieved from the Internet: &lt;URL:http://www.nal.usda.gov/fnic/dga/rda. pdf&gt; 'retrieved on 2000-09-25! tables</p> <p>-----</p> <p style="text-align: center;">-/--</p>	1-33

☒ Further documents are listed in the continuation of box C

☒ Patent family members are listed in annex

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

Date of the actual completion of the international search

26 September 2000

Date of mailing of the international search report

13. 10. 2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl  
Fax: (+31-70) 340-3016

Authorized officer:

Heezius, A

# INTERNATIONAL SEARCH REPORT

national Application No

PCT/US 00/12300

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 981 844 A (ALEXANDER ET AL.) 1 January 1991 (1991-01-01)  claims column 1, line 29 - line 38 column 2, line 32 - line 41 column 2, line 59 - column 3, line 30	1,2, 12-14, 16,17
T	D.W.L. MA, ET AL.: "Preparation of conjugated linoleic acid from safflower oil" JOURNAL OF THE AMERICAN OIL CHEMISTS' SOCIETY, vol. 76, no. 6, 1999, pages 729-730, XP000830933 Champaign, USA page 730; table 1	1-33
A	US 5 674 901 A (COOK ET AL.) 7 October 1997 (1997-10-07) column 2, line 34 - line 42; claims 1,2	1-33
A	WO 92 21368 A (LIFE SCIENCES TECHNOLOGIES, INC.) 10 December 1992 (1992-12-10) claims 1-3,5,8,10,11 page 6, line 13 - line 31 tables 1,2 examples	1-33

# INTERNATIONAL SEARCH REPORT

international application No.  
PCT/US 00/12300

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 12-33  
because they relate to subject matter not required to be searched by this Authority, namely:  
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest
- ☐ No protest accompanied the payment of additional search fees



FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 12-33 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

-----  
Continuation of Box I.1

Claims Nos.: 12-33

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/12300

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5814663 A	29-09-1998	US 5554646 A	10-09-1996
		US 5760082 A	02-06-1998
		AU 720553 B	01-06-2000
		AU 2218797 A	05-01-1998
		CA 2251563 A	11-12-1997
		EP 0907360 A	14-04-1999
		WO 9746230 A	11-12-1997
		EP 0731699 A	18-09-1996
		JP 2992836 B	20-12-1999
		JP 10508189 T	18-08-1998
		WO 9606605 A	07-03-1996
		AU 2338597 A	05-01-1998
		EP 0909132 A	21-04-1999
		WO 9746118 A	11-12-1997
US 4981844 A	01-01-1991	AU 4425289 A	01-05-1990
		CA 2000150 A	04-04-1990
		WO 9003794 A	19-04-1990
US 5674901 A	07-10-1997	AU 5253596 A	18-12-1996
		EP 0831804 A	01-04-1998
		WO 9638137 A	05-12-1996
		US 6060304 A	09-05-2000
		US 6020376 A	01-02-2000
		US 5856149 A	05-01-1999
		US 5827885 A	27-10-1998
WO 9221368 A	10-12-1992	US 5977073 A	02-11-1999
		AU 2187992 A	08-01-1993
		DE 69230796 D	20-04-2000
		EP 0604433 A	06-07-1994